

# Interrogating a Hexokinase-Selected Small-Molecule Library for Inhibitors of *Plasmodium falciparum* Hexokinase

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Parasites in the genus *Plasmodium* cause disease throughout the tropic and subtropical regions of the world. *P. falciparum*, one of the deadliest species of the parasite, relies on glycolysis for the generation of ATP while it inhabits the mammalian red blood cell. The first step in glycolysis is catalyzed by hexokinase (HK). While the 55.3-kDa *P. falciparum* HK (PfHK) shares several biochemical characteristics with mammalian HKs, including being inhibited by its products, it has limited amino acid identity (~26%) to the human HKs, suggesting that enzyme-specific therapeutics could be generated. To that end, interrogation of a selected small-molecule library of HK inhibitors has identified a class of PfHK inhibitors, isobenzothiazolinones, some of which have 50% inhibitory concentrations (IC<sub>50</sub>s) of <1 μM. Inhibition was reversible by dilution but not by treatment with a reducing agent, suggesting that the basis for enzyme inactivation was not covalent association with the inhibitor. Lastly, six of these compounds and the related molecule ebselen inhibited *P. falciparum* growth *in vitro* (50% effective concentration [EC<sub>50</sub>] of ≥0.6 and <6.8 μM). These findings suggest that the chemotypes identified here could represent leads for future development of therapeutics against *P. falciparum*.

Malaria is a global health concern, infecting more than 200 million people a year. While human malaria is commonly caused by four members of the genus *Plasmodium*, *P. falciparum* infection is responsible for the most mortality annually. In addition to disease, the burden of malaria has global economic consequences, with an impact in developing nations that results from reduced worker productivity and markedly increased disability-adjusted life years (DALYs), a measure of disease burden as a consequence of mortality and morbidity (World Health Organization [www.who.int]).

The parasite has a complex life cycle, with the intraerythrocytic stage being primarily responsible for pathology. This stage of *Plasmodium* lacks a complete tricarboxylic acid (TCA) cycle, and knockout and inhibitor studies of mitochondrial proteins have suggested that the mitochondrion is not a significant contributor to cellular ATP levels (1, 2). Notably, glucose consumption was found to be increased up to 100-fold in infected erythrocytes (3), and lactate levels were ~20 to 100 times higher than that from uninfected cells (4, 5). These observations suggested that glycolysis was playing a key metabolic role for the parasite during the erythrocytic infection. Supporting this supposition, knockout studies revealed that the hexose transporter responsible for importing glucose was essential to the parasite, and inhibition of glycolysis with glucose analogs rapidly depleted parasite ATP (6, 7).

The first committed step in glycolysis, catalyzed by hexokinase (HK), is the transfer of the γ-phosphoryl group from ATP to glucose. This reaction yields glucose-6-phosphate (G6P), a metabolite with multiple potential fates. First, it can be consumed in glycolysis. Alternatively, if funneled into the pentose phosphate pathway (PPP), the metabolite can serve in the generation of NADPH, which is a key component in the *P. falciparum* antioxidant defense and *de novo* nucleotide triphosphate biosynthesis pathways (8).

The importance of glycolysis to the malaria parasite and the

observation that the single *P. falciparum* HK (PfHK) is predicted to share limited (24%) identity with human glucokinases (HsGlc, or HK IV) suggested that this enzyme could serve as a suitable target for therapeutics (Fig. 1). Here, we describe the characterization of recombinant PfHK. Further, we have interrogated a small-molecule library of known HK inhibitors to identify potential lead compounds that inhibit PfHK. Lastly, we have assessed the antiparasitic activity of these molecules against *P. falciparum* erythrocytic-stage parasites and have found that several are potent antiparasitic compounds.

## MATERIALS AND METHODS

**Chemicals and reagents.** Glucose-6-phosphate dehydrogenase, β-NAD (NAD<sup>+</sup>), ATP, and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA), while phosphoenol pyruvate (PEP), 2-phenyl-1,2-benziselenazol-3(2H)-one (ebselen [Eb]; PubChem substance identifier [SID] 856002) and glucosamine were obtained from VWR International (West Chester, PA). The isobenzothiazolinones and benzamides used (see Table 2) were obtained from the University of Kansas Specialized Chemistry Center (KUSCC).

**Recombinant enzyme purification and assay conditions.** The open reading frame PF3D7\_0624000 (PlasmoDB) for the *P. falciparum* hexokinase (UniProt Q02155) was synthesized for codon optimization (Genescript, Piscataway, NJ), sequenced, and cloned into pQE30 (Qiagen, Valencia, CA). Recombinant PfHK, an ~55.3-kDa protein, was purified, following a protocol developed for heterologous expression and purifica-

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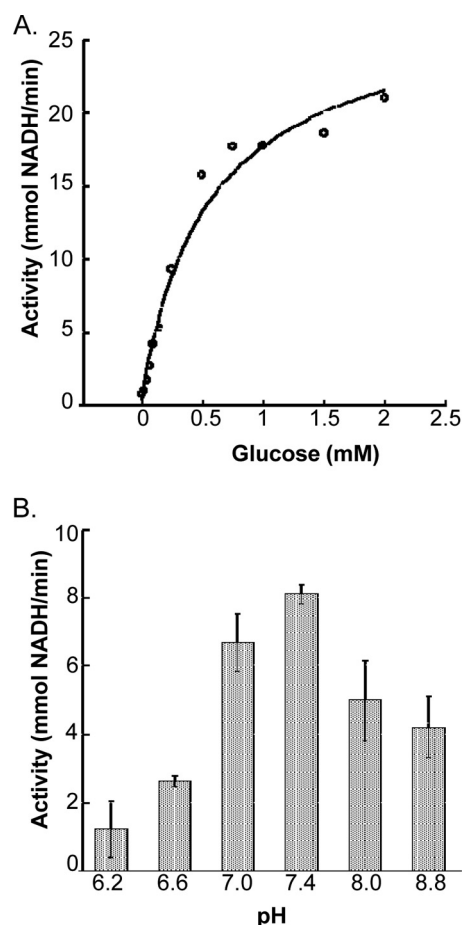
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**FIG 1** Alignments of PfHK, *Trypanosoma brucei* HK1 (TbHK1; Tb427.10.2010), and human glucokinase (HsGlc; ABS31137.1). Sequences were aligned using CLUSTAL 0 (1.1.0) software. The “\*” symbol indicates completely conserved residues, while the colons (:) mark residues that share properties (>0.5 in the Gonnet PAM250 matrix). Periods (.) indicate residues that fall at <0.5 in the Gonnet PAM250 matrix, indicating weak similarity. The boxed residue is the conserved catalytic base (Asp241 in PfHK).

**Hexokinase inhibitor library.** The collection of small molecules described here as a “selected small-molecule library of HK inhibitors” was originally developed, identified, and characterized as part of a high-throughput screening (HTS) campaign targeting hexokinase 1 from the African trypanosome, *Trypanosoma brucei* (TbHK1). This screen was completed at the Pittsburgh Molecular Libraries Screening Center (PMLSC) using a 220,233-compound library that was made available as

The dose-response curves for compounds that caused >50% growth inhibition at 10  $\mu$ M were pursued in a 96-well plate format using an asynchronous parasite culture. The starting parasitemia was 1%. Fifty percent inhibitory concentrations (IC<sub>50</sub>s) were determined by scoring parasite growth in three separate wells over a 2-fold dilution series starting



**FIG 2** Effects of glucose concentration and pH on PfHK activity. (A) PfHK (200 ng) activity in response to increasing glucose concentration. (B) The pH profile of PfHK (200 ng) activity. Three different buffers with buffering capacity in the range of pH tested were used for the assay: piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.2, 6.5, and 7.0), triethanolamine (pH 7.4), and Tris (pH 8.0 and 8.8). Reactions were carried out in triplicate, and standard deviations are indicated.

at 40  $\mu$ M for each compound. Percent inhibition was calculated by comparison to parasites grown in the DMSO controls from each plate. Chloroquine was screened as a positive control starting at 1  $\mu$ M. Averages for the triplicates were calculated, and dose-response curves were fitted and  $IC_{50}$ s determined using the software program Prism (GraphPad Software).

## RESULTS

While the importance of glycolysis to *P. falciparum* during mammalian infection has been established (3–5), the enzymes involved in the process have not been subjected to in-depth characterization. This is likely due to the difficulty of classically purifying the parasite components from the complex milieu of the red blood cell contents (which includes many related host activities). Additionally, the A-T-rich nature of the *Plasmodium* genome has historically made production and analysis of recombinant enzymes difficult.

Indeed, our initial forays into expression of PfHK in *E. coli* were unsuccessful despite repeated efforts using a battery of expression vectors and bacterial cell lines. Expression was finally achieved using a synthetic PfHK gene optimized with a codon bias

**TABLE 1** Kinetic characterization of rPFHK<sup>a</sup>

HK	$k_{cat}$ ( $\text{min}^{-1}$ )	Apparent $K_m$ (mM)	
		Glucose	ATP
rPFHK	$1.2 \times 10^4$	$0.62 \pm 0.06$	$0.66 \pm 0.08$
rTbHK1 <sup>b</sup>	$2.9 \times 10^4$	$0.09 \pm 0.02$	$0.28 \pm 0.1$

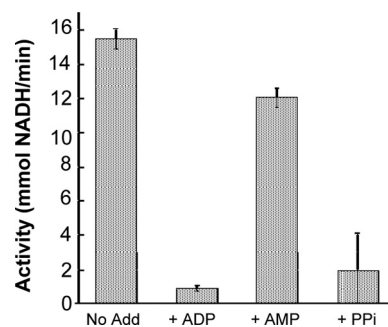
<sup>a</sup> rPFHK and rTbHK1, recombinant PfHK and TbHK1.

<sup>b</sup> Data from reference 9.

that reflected those codons employed by *E. coli*. The recombinant enzyme, which was purified to ~95% homogeneity (as determined by Coomassie staining of an SDS-PAGE gel), displayed Michaelis-Menten kinetics when substrate levels were increased (Fig. 2A) and had apparent  $K_m$  values for glucose and ATP ( $0.62 \pm 0.06$  and  $0.66 \pm 0.08$  mM, respectively) similar to those from yeast HK and *T. brucei* TbHK1 (Table 1) (9). The PfHK  $k_{cat}$  value was  $1.2 \times 10^4 \text{ min}^{-1}$ , similar to that reported for a recently characterized *T. brucei* HK1 ( $2.9 \times 10^4 \text{ min}^{-1}$ ) (9). PfHK had an affinity for  $\text{MgCl}_2$  (apparent  $K_m = 2.7 \pm 0.4$  mM) similar to that of TbHK1 (apparent  $K_m = 0.92 \pm 0.21$  mM). PfHK lacked detectable activity when  $\text{CaCl}_2$  or  $\text{MnCl}_2$  was included in place of  $\text{MgCl}_2$  in the standard assay. The optimal pH for enzyme activity was found to be about pH 7.4, with enzyme activity reduced ~50% by a 1.2 pH unit change in either the basic or acidic direction (Fig. 2B).

The enzyme lacked detectable activity when alternative phosphoryl donors, including UTP, GTP, CTP, TTP, ITP, and  $\text{PP}_i$ , were tested. Notably, ATP at concentrations greater than 3.5 mM was inhibitory to the reaction (data not shown). Products also inhibited the enzyme, with inclusion of ADP (10 mM) in the reaction inhibiting the enzyme ~94% (Fig. 3).  $\text{PP}_i$  was also inhibitory, while AMP only modestly (~22%) inhibited PfHK at 10 mM. G6P, another product of PfHK, inhibited the enzyme ~80% at 10 mM.

**Identification of small-molecule inhibitors of PfHK.** The completion of an HTS for inhibitors of *T. brucei* hexokinase 1 (TbHK1) yielded several scaffolds that have since been further elaborated to resolve structure-activity relationships against that enzyme (12). We have taken advantage of the reagents and assays developed therein to interrogate this selected small-molecule library of HK inhibitors for PfHK inhibitors. These compounds have been rigorously characterized. For example, they do not inhibit the coupled reporter enzyme, are typically poor inhibitors of human glucokinase, have variable activity against TbHK1, and are



**FIG 3** Effects of phosphoryl-bearing compounds on PfHK activity. PfHK (200 ng) activity was assessed in the presence of 5 mM ATP supplemented with ADP,  $\text{PP}_i$ , or AMP (all at 5 mM). Assays were performed in triplicate as described in Materials and Methods, and standard deviations are indicated.



minimally toxic to a number of mammalian cell lines (including A549, IMR-90, HeLa, and MDA-MB-231 (12)).

Of the 16 hits obtained from the HTS effort against TbHK1, two groups were pursued as scaffolds for analog development against TbHK1. Our interest in the first analog group, which was based on five representative isobenzothiazolinones found in the TbHK1 HTS, was particularly piqued because one analog, SID 92126115, had been previously identified in a screen performed by GlaxoSmithKline of more than 2 million compounds for molecules with anti-*Plasmodium* activity (<http://www.ebi.ac.uk/chemblntd>) (13). SID 92126115, which inhibited parasite growth 97% at 2  $\mu$ M, was a potent PfHK inhibitor ( $IC_{50} = 0.16 \pm 0.04$   $\mu$ M) (Table 2). Other isobenzothiazolinones lacking the halogen substituents (SID 85747718 and SID 17387000) had slightly increased  $IC_{50}$ s, though substitution of the sulfur atom with a selenium atom (to yield SID 856002, ebselen [Eb]) increased potency of inhibition, a trend similar to that seen when Eb inhibition of TbHK1 was compared to that seen with SID 17387000. Interestingly, Eb and SID 17387000 inhibition was reversible by dilution (Fig. 4 and data not shown), which is in contrast to the irreversible inhibition observed with TbHK1 (M. T. Harris and J. C. Morris, unpublished observation). The reducing reagent dithiothreitol (DTT) was able to prevent but not reverse Eb inhibition of PfHK, likely as a consequence of the preventative DTT binding directly to Eb to block its interaction with the enzyme (14). Last, the presence of the sulfur or selenium atom was important for inhibition of the enzyme, since substitution with either a carbon or oxygen atom at that position (SID 87225568 and 85752767, respectively) ablated inhibition.

We also explored inhibition of PfHK with members of a second scaffold identified in the TbHK1 HTS, based around the benzamidobenzoic acid identified in the screen (SID 93575727). Further development of this class of compounds has yielded improved inhibitors of TbHK1, including the probe ML205 (SID 99437306) (Table 1) (11). However, against PfHK, representatives of this class were either poor inhibitors (ML205 was  $\sim 40\%$  inhibitory at 10  $\mu$ M) or lacked detectable activity against the enzyme.

**PfHK inhibitors are toxic to *P. falciparum*.** Glycolysis is important to *P. falciparum* during the erythrocytic infection, suggesting that inhibitors of the enzyme could be promising lead compounds for therapeutic development. To explore this possibility, we grew cultured *Plasmodium* parasites in the presence of compound and monitored cell density after 72 h. Both the isobenzothiazolinones and benzamidobenzoic acid-based compounds were tested in this assay, with 50% effective concentrations ( $EC_{50}$ s) determined for those that inhibited cell growth  $>50\%$  at 10  $\mu$ M (Fig. 5 and Table 2). Compounds from the isobenzothiazolinone group included two of the most potent antimalarial compounds, SID 92126115 and SID 85747718. While all of the compounds that inhibited PfHK were toxic to parasites, one molecule (SID 85742034) was toxic to parasites while not inhibiting PfHK. With the exception of SID 87225569, the antiparasitic compounds had  $EC_{50}$ s that were 4- to 680-fold higher than the PfHK  $IC_{50}$ s. This discrepancy could result from the inability of the molecules to reach their target as a consequence of limited RBC and/or parasite cell permeability or trafficking. The identified PfHK inhibitors had minimal impact on human cell lines, with  $EC_{50}$ s of  $>12.5$   $\mu$ M (12), suggesting at least 20-fold-greater toxicity toward parasites for the most potent compound (Table 2).

## DISCUSSION

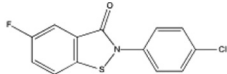
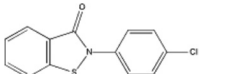
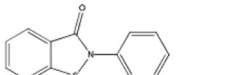
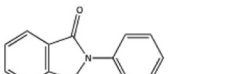
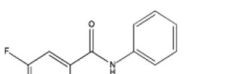
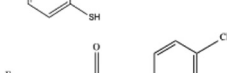
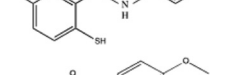
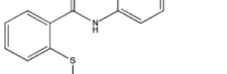
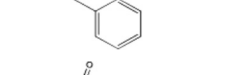
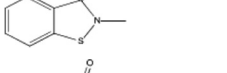
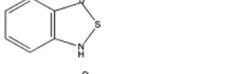
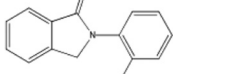
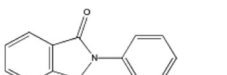
The intraerythrocytic malaria parasite requires extracellular glucose for survival, largely due to the significant consumption of the hexose as a consequence of ATP generation by glycolysis. Inhibitors of this pathway have demonstrated potent antimalarial activity. These include small molecules that interfere with hexose transport (15), as well as glucose analogs like 2-deoxyglucose (6, 16).

PfHK, the first enzyme in glycolysis, has heretofore been considered an interesting potential target but has not been exploited due to an inability to produce active recombinant protein for *in vitro* characterization (17). Using an *E. coli* codon-optimized gene, we have for the first time generated active PfHK. Characterization of PfHK revealed it to share a number of features with other HKs, including the finding that the reaction products G6P and ADP were inhibitors of the enzyme, suggesting the *Plasmodium* protein may be regulated via the feedback mechanism described for vertebrate HKs (18). This observation was somewhat surprising given that an HK from the related apicomplexan parasite *Toxoplasma gondii* (which is 44% identical to PfHK) is not sensitive to substrate inhibition (19). PfHK is also kinetically similar to other HKs, having glucose, ATP, and  $MgCl_2$   $K_m$  values similar to those of HKs from other systems, including *T. brucei* and *Homo sapiens*. The PfHK  $K_m$  value for glucose was similar to that described by Roth from activity associated with infected RBC lysates (620  $\mu$ M and 431  $\mu$ M, respectively) (20). These values are  $\sim 5$ -fold higher than the  $K_m$  reported from HK activity associated with uninfected RBC lysates, suggesting that the host enzyme should outcompete the parasite enzyme for glucose under limiting conditions. However, glucose transport in the intraerythrocytic parasite is highly efficient, with the concentration of the substrate inside the parasite rapidly reaching levels higher than that found in the external medium (15).

The second PfHK substrate, ATP, is required for activity but can also be an inhibitor of the enzyme. PfHK has a  $K_m$  value for ATP well below the intraerythrocytic ATP concentration ( $\sim 3$  mM) and is inhibited at ATP levels approaching those found in the host cytoplasm. While we anticipate that PfHK utilizes a pool of ATP distinct from that found in the host cytoplasm, there could be conditions under which the parasite has generated sufficient ATP to satisfy its needs. To continue to synthesize ATP could be damaging to the host cell, either directly as a consequence of usurping host glucose or by depletion of host purines required for ATP synthesis, since the parasite cannot synthesize purines and must salvage them from the host. The finding that ATP is a regulator of HK activity is not novel among HKs (21), though its role in the malaria parasite remains unclear.

Taking advantage of a preexisting library of small molecules designed around two scaffolds known to inhibit a trypanosome HK, we searched for inhibitors of PfHK. The two enzymes share limited sequence identity (28%) (Fig. 1), but nevertheless we found that members of one of the two classes of analogues, the isobenzothiazolinones, were more potent against PfHK than against TbHK1 (Table 2). Additionally, the isobenzothiazolinone analog Eb was extremely potent against PfHK ( $IC_{50} = 0.01 \pm 0.00$ ). Eb can promiscuously modify cysteine residues, and this nonspecific interaction is known to be the mechanism of its inhibition of some enzymes, such as human indoleamine 2,3-dioxygenase (22). However, site-directed mutagenesis of TbHK1 cys-

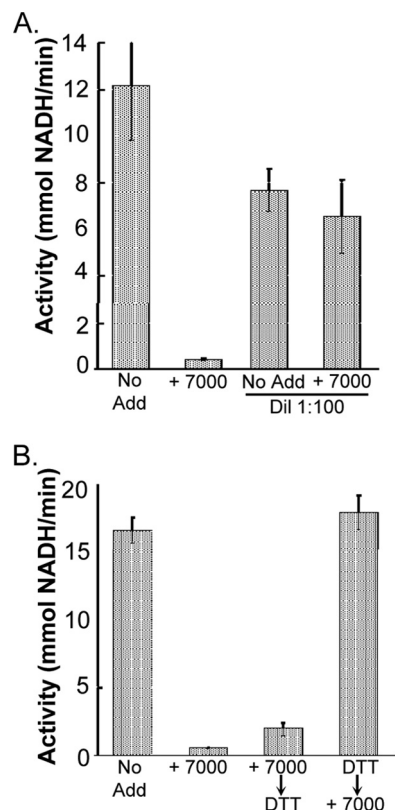
TABLE 2 Sensitivities of PfHK to TbHK1 inhibitors

PubChem SID	Structure	PfHK IC <sub>50</sub> (μM)	TbHK1 IC <sub>50</sub> (μM) <sup>a</sup>	<i>P. falciparum</i> EC <sub>50</sub> (μM)
92126115		0.16 ± 0.04	3.8 ± 0.40	0.62 ± 0.06 <sup>b</sup>
85747718		0.27 ± 0.01	1.1 ± 0.34	1.1 ± 0.39
17387000		0.23 ± 0.03	2.0 ± 0.50	5.2 ± 0.23
856002 (ebselen)		0.01 ± 0.00	0.05 ± 0.03	6.8 ± 1.25
87225569		3.1 ± 0.28	>25	4.4 ± 0.36
160644636		ND <sup>c</sup>	ND	1.8 ± 0.72
87325999		>10	>25	ND
85742034		>10	>25	5.1 ± 0.82
85786735		>10	>25	ND
87225568		>10	>25	>10
85752767		>10	>25	>10
93575727		>10	9.3 ± 3.5	>10
99437306		>10	0.98 ± 0.07	>10

<sup>a</sup> Data from references 11 and 12.<sup>b</sup> Identified in a GSK screen as inhibiting 3D7 *P. falciparum* growth 97% at 2 μM (<http://www.ebi.ac.uk/chemblntd> [13]).<sup>c</sup> ND, not determined.

teines, including the two conserved in PfHK (residues 273 and 398 in PfHK), did not alter TbHK1 sensitivity to Eb inhibition, indicating that either cysteine residues are not involved in Eb inhibition or multiple cysteines must be bound in order for inhibition to

occur. Unlike the trypanosome enzyme, PfHK inhibition by Eb and the isobenzothiazolinone SID 17387000 was reversible by dilution, suggesting that the inhibitors interact with the two enzymes differently.

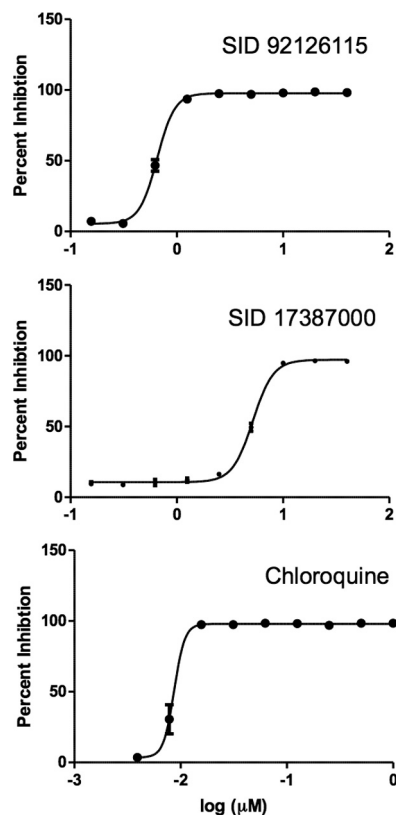


**FIG 4** Characterization of PfHK inhibition by SID 17387000. (A) Dilution after inhibition with SID 17387000 recovers enzyme activity. PfHK (200 ng) was incubated with SID 17387000 (0.5  $\mu$ M) or buffer (10 min, on ice) before addition of other assay components, resulting in an  $\sim$ 100-fold reduction in the SID 17387000 concentration. (B) Addition of the reducing agent DTT (100 mM) before but not after addition of SID 17387000 (0.5  $\mu$ M) prevented PfHK inhibition. Standard deviations are indicated.

Previous research has demonstrated that Eb is toxic to *P. falciparum*, with  $EC_{50}$ s similar to those found in our study (14  $\mu$ M and 6.8  $\mu$ M, respectively) (23). Eb may have a polypharmacological impact on the parasite, given the likely reactivity of the selenium atom with cellular cysteines. However, it is nontoxic for humans and is currently in phase III clinical trials for the treatment of stroke survivors (Stroke Trials Registry Home Page as of 27 February 2013), suggesting that the consequences of off-target effects in the human host may be tolerable.

The isobenzothiazolinone SID 92126115 was previously identified in a screen performed by GlaxoSmithKline of more than 2 million compounds for molecules with anti-*Plasmodium* activity, inhibiting parasite growth 97% at 2  $\mu$ M (<http://www.ebi.ac.uk/chemblntd>) (13). SID 92126115 was a potent PfHK inhibitor, with an  $IC_{50}$  of  $0.16 \pm 0.04$   $\mu$ M. Isobenzothiazolinone inhibitors of PfHK were also effective at inhibiting the growth of cultured parasites, with  $EC_{50}$ s below 10  $\mu$ M. The  $EC_{50}$ s were higher than  $IC_{50}$ s found for PfHK, likely due to issues with cell permeability and off-target interactions. With the exception of SID 85742034, compounds that did not inhibit PfHK did not kill parasites.

In conclusion, several observations support the value of PfHK as a therapeutic target. First, the single-copy gene product shares limited (26%) identity with the human-equivalent enzyme. Second, the enzyme is responsible for catalyzing the first reaction in



**FIG 5** Isobenzothiazolinones inhibited the growth of *P. falciparum* 3D7 parasites in a dose-dependent manner. Twofold dilution series of the compounds SID 92126115 and SID 17387000 were incubated with parasites, and percent inhibition was calculated by comparison to results for parasites grown in the DMSO controls from each plate. Chloroquine was included as a positive control. Averages for experiments performed in triplicate were calculated and  $EC_{50}$ s were determined using Prism (GraphPad Software), with standard deviations indicated.

two critical pathways, glycolysis and the PPP, and is likely indispensable for parasite viability. Last, inhibitors of the enzyme can inhibit parasite proliferation, as demonstrated here. Together, these findings suggest the value of further consideration of the isobenzothiazolinone scaffold, as well as identification of additional new chemotypes, as a potential lead in therapeutic development.

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